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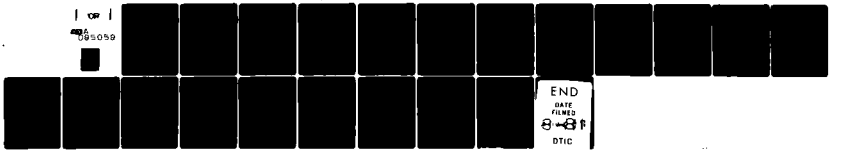

RELATION OF CELL WALL TO THE VIRULENCE OF SHIGELLA FLEXNERI

Final Comprehensive Report

Laurence M. Corwin

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Most of the work ~~from this~~ laboratory has been devoted to trying to understand why the avirulent mutant of S. flexneri 2a (24570), which is antigenically identical to its virulent parent (M-42-43) is nevertheless unable to penetrate the intestinal epithelium. It was found that the strains were different in several characteristics of the cell wall. First, the 24570 was much more resistant to sodium lauryl sulfate, an anionic detergent, than the virulent strain, M4243. It has also been shown that the avirulent strain is four times as electronegative, but this was dependent on the presence of

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20. calcium ions. Apparently the electronegativity has made the avirulent strain resistant to other agents such as EDTA and lysozyme.

Other studies have shown that the avirulent mutant is deficient in the enzyme, glycerol kinase. This enzyme has shown to be genetically linked to the expression of electronegativity. It was thought that in Shigella, a cell wall mutation may be affecting the expression of glycerol kinase, hence the relation to electronegativity. This was given more credence when it was demonstrate that the uptake of Krebs cycle acids was severely curtailed.

Finally, work was begun ~~before the termination~~ of the contract to indicate that fluorescent probes may be useful to detect differences between virulent and avirlent strains of Shigella.

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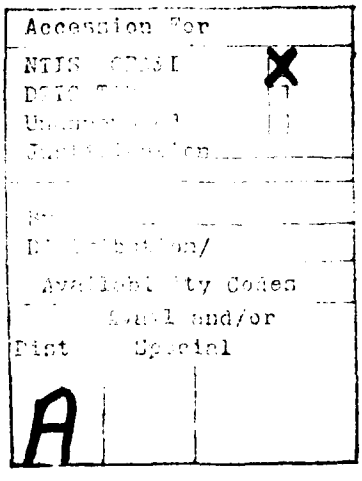

Summary

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Other studies have shown that the avirulent mutant is deficient in the enzyme, glycerol kinase. This enzyme was shown to be genetically linked to the expression of electronegativity. It was thought that in Shigella, a cell wall mutation may be affecting the expression of glycerol kinase, hence the relation to electronegativity. This was given more credence when it was demonstrated that the uptake of Krebs cycle acids was severely curtailed.

Finally, work was begun before the termination of the contract to indicate that fluorescent probes may be useful to detect differences between virulent and avirulent strains of Shigella.

Background

a. Basis - Genetic Studies of Virulence of Shigella

The classical method of studying bacterial virulence is to compare the properties of virulent and avirulent strains. The fact that two strains differ in virulence is easily established, but discovery of the specific genetic marker involved is less easily obtained. It is necessary to have some observable character distinguishing the two strains in vitro and to be able to prove that this is invarably associated with virulence (Burrows ,1962). Microbial genetics and studies of biochemical mechanisms, i.e., studies of enzymes, metabolic character , etc., have indicated many markers associated with virulence but few, if any, have been shown to be virulence determinants. One must be constantly aware that if virulence is due to many factors, an avirulent strain may possess all but one of these factors (Smith, 1968).

The first method of distinguishing virulent from avirulent strains was colonial variation (Cooper et al., 1957; Kerekes, 1962; Schneider and Formal, 1963). The spontaneous mutation of the translucent colonial form of the virulent Sh. flexneri 2a strain M-42-43 to its avirulent opaque form, strain 24-70, occurs at a rate of 1 in 10^4 cells (Falkow et al., 1963). Opaque variant 24570 is avirulent in the guinea pig, does not cause keratonconjunctivitis in the guinea pig eye, and is not able to penetrate HeLa cells.

Although various investigators have described colonial variants of Shigella strains which are associated with a loss of virulence for various animal hosts, Formal et al. (1965 a) have shown that the disappearance of virulence may differ from variant to variant. Using three spontaneous avirulent mutants of Sh. flexneri 5 strain M90 exhibiting the same change in colonial morphology, only one could be restored to virulence by mating with Escherichia coli. Hybrids with the colonial morphology of M90 were not virulent. Thus, in Sh. flexneri 6, alterations in more than one gene can alter colonial morphology, but only one of these is associated with virulence.

The genetic homology between E. coli K12 and Sh. flexneri is about 85% (Falkow and Formal, 1969; Baron et al., 1968). Hybrids of Sh. flexneri 2a containing segments of E. coli chromosome covering 50% of the genome revealed only one region located between rhamnose and xylose genes essential for virulence in this strain (Falkow et al., 1963). Subsequent investigations show that the loss of virulence of some of these hybrids was caused by an inability of the bacteria to multiply sufficiently in epithelial cells after penetration (Formal, 1965a).

It is also possible to restore virulence to an avirulent strain. When mal$^+$ hybrids were selected in a cross between E. coli W-1895 (Hfr C) mal$^+$ and an avirulent mutant of Sh. flexneri 5 mal$^-$, most of these had regained virulence.

Krishnapillae and Baron (1964) were able to show in Salmonella abony the existance of two genes associated with the loss of virulence. One maps next to the str A marker and the other next to met B. From their evidence they proposed that each determinant by itself would allow phenotypic expression of partial avirulence, but both are presumably necessary for the expression of complete avirulence. These markers are generally compatible with the location of the avirulent determinant of E. coli as determined by Falkow et al. (1963).

Formal et al, (1971) have recently succeeded in identifying a genetic locus on the Sh. flexneri chromosome which controls epithelial cell penetration, the kcp A locus. It is located near the pur E locus. It would seem to be at a site quite far away from the locus affected by the strain 24570 mutation. E. coli K12 hybridized with the kcp A$^+$ allele does not evoke keratoconjunctivitis; therefore other Shigella genes must be involved in host cell penetration.

The streptomycin marker (str A) has also been associated with virulence (Petrovskaya and Licheva (1970a, b). Strains of shigellae and salmonellae resistant to streptomycin are often avirulent (Falkow et al., 1963; Krishnapillae and Baron, 1964).

Biochemical Studies on Virulence of Shigella

Studies on the biochemical basis for virulence of Shigella must take into account the sequence of biological events required: a) penetration of the intestinal epithelial cells, b) entrance into the lamina propria, followed by a period of intensive bacterial multiplication, c) destruction of the host cell (LaBrec et al., 1964; Formal et al., 1965b, 1966). The study of penetration has made use of an isogenic pair of Shigella flexneri 2a, a virulent strain, M-42-43, and a spontaneous avirulent mutant, 24570. The latter is one of several alterations in Sh.flexneri rendering it unable to penetrate. For example, one can alter the O antigen makeup of Shigella by making hybrids with E. coli, selecting for this his locus. The incorporation of the E. coli O-8 repeat unit from E. coli will prevent Shigella flexneri from penetrating (Gemski et al., 1971). Another hybridization at the kcp A locus near pur E will also prevent penetration (Formal et al., 1971). Nothing is known about the nature of kcp A mutation. It shall be brought out in the following that the 24570 spontaneous mutation occurs at a site different from the kcp A locus and the his linked O antigen site.

b. Work by Investigator

Most of the earlier work from this laboratory has been devoted to trying to understand why the avirulent mutant (24570) which is antigenically identical (LaBrec et al., 1964) to its virulent parent (M-42-43) is nevertheless unable to penetrate the intestinal epithelium. The research was designed to test the hypothesis that the avirulent strain, 24570, does not attach to the host cell because of an increased electronegative charge. This is usually tested by measuring the rate of uptake of the cationic dye methylene blue, washing the bacteria free of external methylene blue, and finally lysing of the bacteria with sodium lauryl sulfate (SLS) to release the methylene blue for measurement. It was found that the experiment could not be done properly because of the inability to lyse the avirulent strain with SLS (Corwin et al., 1971). This was confirmed by rate studies showing that the virulent M4243 is lysed four times as rapidly as the avirulent 24570 (Corwin et al., 1973). The conditions for lysis by SLS included growth of the strains in Luria broth with 5mM Ca^{++}. The lysis was carried out in phosphate buffer. If Tris buffer was used instead no lysis of either strain was observed by SLS. In the absence of Ca^{++}, both strains were ly ed to a much greater extent, but the 24570 was still lysed more slowly. It was also found that the 24570 was more resistant to lysis by EDTA and lysozyme. Thus the mutation to avirulence has resulted in a stabilization of the wall against lysing agents.

Returning to the hypothesis of electronegativity as an explanation for non-attachment of the avirulent mutant, charge was now tested by microelectrophoresis. By this technique, it was monstrated that in fact the 24570 was up to four times more negative that the virulent M-4243 (Corwin and Talevi, 1972). However, such a difference could only be demonstrated if the strains were grown in the presence of 5mM Ca^{++}. Thus the increased negativity of 24570 is only seen when grown in the presence of a cation. This anomaly could be explained by either of two hypotheses. The first involves an alteration in the conformational structure of the cell wall by chelating cations, for example by ions such as Ca^{++} which bring negatively charged components to the surface. When the calcium ions are removed by the electric field or by EDTA, the negative charge remains. The alternative is that calcium ions loosen the bonds of some surface structure, allowing the release of macromolecules into the medium and uncovering a more negatively charged layer.

It was found that EDTA would increase the charge of the 24570 when grown in Luria broth and Ca^{++}, but would not affect the M-4243 charge under the same conditions (Corwin and Talevi, 1972). Thus, the release of lipopolysaccharide (LPS) and protein from the two strains by EDTA was tested. In fact more protein and LPS was released from the virulent strain than the avirulent. Thus the increased charge of the avirulent strain does not appear to be explainable by the uncovering of a negative layer after release of macromolecules from the more superficial layers.

Other studies have shown that the avirulent mutant is deficient in the enzyme glycerol kinase making it unable to ferment glycerol (Kim and Corwin, 1973). This observation gave the first genetic handle to study some of the relationships between the several alterations in characteriistics of the avirulent mutant from that of the virulent Shigella strain. It was discovered that electrophoretic charge always increased upon the loss of glycerol kinase. The mutation rate to glycerol kinase negative strains was about 1 in 10^4, which is about the same rate as that of the change in colonial morphology from translucent colonies to the opaque avirulent colonies. Interestingly the ability to penetrate was lost by only about half of the glycerol kinase negative strains indicating that more than one gene must be involved.

There are two particular findings which have led to the establishment of the fluoresecent technique used to distinguish penetrating from non-penetrating mutants of Shigella. The first is the interesting correlation made by Kerekes (1973) between acid agglutination of the bacteria and their virulence. Saline suspensions of virulent 24 hr. cultures showed agglutination in one or more tubes of a buffer solution in the range of pH 1.6-4.9, while the majority of avirulent S-form culture either did not agglutinate at all or agglutinated in the narrow range of pH from 1.0-1.4. The virulent S. flexneri cultures failed to agglutinate after washing in ethanol or cetylpyridinum chloride. Heating (100°) caused a loss of agglutinability in most virulent strains, while some avirulent strains became agglutinable after heat treatment. Nakamura (1967) has shown that S. flexneri 1b virulent strains do not agglutinate and Kerekes has explained this as being due to the inability to maintain homogeneous colonial cultures in that strain.

The other finding, by Kim and Corwin (1973), involved the oxidation and uptake of Krebs cycle acids such as succinate. There is little difference in these parameters between the virulent M-42-43 and avirulent 24570 strains of S. flexneri 2a, if the cells are harvested from logarithmic growth phase and/or if they are grown in static culture.

When cells are grown overnight with vigorous shaking the ability of the avirulent 24570 strain to oxidize succinate as well as to transport it is greatly curtailed. Under these conditions there is a resultant 3 to 4 fold difference between the 24570 strains and the M-42-43, whose performance in these characteristics is unaltered.

c. Current work

During the past year, the use of fluorescent dyes to probe the structure of the cell wall was begun with the hope of uncovering differences between virulent and avirulent strains. The method involves the introduction of small molecules into the membrane that have characterisitc fluorescent properties in a given environment (Radda and Vanderkooi, 1972). Fluorescent measurements can reflect the effect of its microenvironment on a particular probe. For example, 1-anilinonaphthalene-8-sulfonate (ANS) has been shown to be located at polar-non-polar interfaces (i.e., lipid-protein, lipid-water or lipid-protein-water contacts), while others, which have no polar groups, such as N-phenyl-1-naphthylamine (NPN) locate in hydrophobic interiors such as the hydrocarbon regions of lipids (Waggoner and Stryer 1970). Much of our time has been spent using NPN as the fluorescent probe. NPN, in a hydrophobic environment, exhibits an emission maximum below 400 nm as compared to 460 mm in water, and has a much higher quantum yield. Thus, for NPN, upon excitation at 340nm, the maximum emission was at 466 nm when the solvent was saline, 461nm for water, 420nm, for methanol and 393 for benzene, while the emission peak heights (in arbitrary units) were, respectively, 7.4, 11.0, 247.5 and 720.0. When equal concentrations of cells were exposed to equal amounts of NPN, the M-42-43 showed a much more rapid binding, as indicating by the emission wave length shift from 460 to 405 nm and by the increase in peak height, than did the 24570. At any given concentration of NPN, the quantum yield upon binding to M-4243 is higher than that to 24570, until saturation is reached. With a given concentration of NPN, where the quantum yield differed by a factor of two, we explored the above differences as a function of growth conditions, cation concentrations, etc.

The greater and more rapidly achieved quantum yield for M-4243 than for 24570 was observed when the bacteria were grown to stationary phase in the absence of Ca^{++} with 0.2% glucose in Luria broth. These probe results were correlated with the spontaneous release of LPS into the test fluid. In three determinations the M4243 released 2.2 + 0.5% of its LPS during the course of the experiment whereas the avirulent 24570 strain released 0.7 + 0.2%.

It was considered possible that the loss of LPS permitted the NPN to penetrate more easily into the hydrophobic interior of M-4243. Strong doubt was cast on this interpretation when binding of NPN to rough mutants or both strains was tested. These mutants were isolated by growing the strains in the presence of rabbit antiserum to the strains. In these strains the O-antigens are unable to bind to the LPS core regions. It was found that NPN was bound much more readily to the rough M mutant than the rough O mutant, results similar to that observed with the parent strains. Thus it would seem that the O-antigen is not involved with the NPN-binding differences observed with the two strains. Other observations also confirm that, although NPN can bind to purified Shigella LPS and give rise to an emission maximum at 404nm, the observed quantum yield is far less than that of the whole bacteria carrying an equivalent amount of LPS.

Studies were then carried out to determine the conditions necessary to produce differences in NPN binding by the two strains. The differences between the Shigella strains were observed with stationary phase cells grown in Luria broth with 0.2% glucose and no added Ca^{++}. Cells harvested from logarithmic phase do not show the difference. The avirulent cells under these conditions bind NPN as well as the M4243 which is relatively unchanged by growth conditions. The presence of 5mM Ca^{++} decreases the binding of NPN to M4243 so that little difference in NPN-binding to the two strains occurs. Finally, when the cells are grown in high glucose concentrations (2% vs. 0.2%) binding of NPN to both increased to such an extent that no difference between the strains is seen. It would seem therefore that internal binding sites for NPN exist in both strains, but that the channels to these binding sites may vary in the two strains under certain conditions. Treatment of the cells with EDTA or with EGTA after growth in 0.2% glucose without added Ca^{++} was also t ed While the overall quantum yield decreased for both strains, the relative values, ie., the greater yeild for M-4243 than for 24570, were maintained.

Fortunately our recent work included some important controls which made much of the published information on NPN binding, as well as our own earlier work, suspect. The first observation came after the cells labeled with NPN were centrifuged and [illegible] in fresh media. Some 80% of the fluorescence was lost. NPN exhibits an enormous increase in quantum yield when entering a hydrophobic environment from an aqueous one. Since this increase was largely lost by simple centrifugation of the labelled cells, it was speculated that most of it was due to NPN micelles loosely attached to the bacteria.

It seems unlikely, however, that a hydrophobic interaction between NPN and the cells would be so easily broken. Another serious flaw in the NPN experimentation was our finding that NPN binds ubiquitously to glass, to siliconized glass, and to plastic (e.g., Teflon stirring bars, polypropylene or polycarbonate tubes). Furthermore, prelabeled bacteria lose some of their bound NPN when transferred to a new glass tube. Both of these observations are manifestations of the great affinity of NPN for surfaces, and could be verified by benzene extraction of the bacteria as well as by benzene washing of tubes, flasks, stirring bars after incubation of the bacteria. All in all these properties of NPN made absolute quantitative interpretation of binding studies very difficult indeed. It is not, however, necessary to discard our results inasmuch as consistent differences between benzene extracts of virulent and avirulent cells were obtained by identical treatment of the bacteria (Table 1).

The data in Table 1 indicate that EDTA treatment of the bacteria greatly increases the available NPN binding sites in the virulent M-42-43 but insiginficantly affects the non-invasive 24570. Thus, while no significant binding differences between the strains exist at pH 7.0 without EDTA treatment, M-42-43 binds significantly more NPN than 24570 after such treatment. Since NPN is a hydrophobic probe, it is to be expected that either the hydrophobic regions are changed in this mutation or that these results are a reflection of the relative resistance of 24570 to EDTA treatment. In any event, this protocol serves to distinguish between the two strains.

Table 1

NPN-binding to S. flexneri 2a Strains[1]

Strain	Medium[2] Ca^{++}	Fluorescence[3] + SEM + 0.1mM EDTA	-EDTA
24570	-	109 ± 27 (4)[4]	71 ± 26 (4)
24570	+	114 ± 31 (5)	83 ± 44 (5)
M-42-43	-	283 ± 59 (5)	60 ± 18 (5)
M-42-43	+	381 ± 91 (5)	64 ± 32 (5)

1. Harvested bacteria were resuspended in 0.1mM EDTA ph 7.0 for 15 minutes. They were recentrifuged ans washed once in saline. They were then resuspended to a concentration of 2 X 10^8 bacteria/ml and exposed to 0.5 ug NPN for 1 hr.

2. Growth medium was Luria broth + 5mM $CaCl_2$ and included 0.2% glucose.

3. The labelled cells were centrigued, washed, and the washed pellet extracted with a quantity of benzene equivalent to the initial volume of cell suspension. The fluorescence was measured at 395 mm after exitation at 340 mm.

4. Numbers in parentheses equal the number of samples.

Nevertheless we have since turned to ANS with which the surface adsorption problems encountered with NPN do not exist. ANS has a polar sulfonate group, allowing it to attach to hydrophobic-hydrophilic interfaces. The initial findings, reported last year, were that ANS did not appear to bind to the bacteria to any utilizable extent at pH 7.0. This was thought to be attributable to the negative charge of the bacteria at neutral pH. This hypothesis, coupled with the acid agglutinability studies of Kerekes (1973) outlined above led us to try our binding studies with ANS at pH 3.3 in an 0.1 M glucine-HCl buffer. The results are detailed in table 2. It should be noted that, at present, the labelling detected at pH 7 with ANS is so near the experimental error that we have pursued our studies at pH 3.3 where the binding of ANS is much greater. All values reported in table 2 for fluorescent intensity are at a higher scale expansion, 9X greater than that in table 1; this relfects 1) observation that the fluorescence of NPN in non-polar solvents such as benzene is extremely high, 2) possible differences in inherent quantum yield between ANS and NPN, and perhaps 3) some absolute differences in NPN and ANS binding. No effort has yet been made to sort out the contributions of each of these possibilities.

At pH 3.3, cells grown overnight in static culture labelled intensely with ANS. There was, however, very little difference between the virulent and avirulent strains. If the experiment was carried out with bacteria obtained from cultures vigorously aerated in Erlenmeyer flasks, the virulent strain loses about two-thirds of its ability to bind ANS, whereas 24570 is virtually unaffected. This is very different from our observations on succinate oxidation in static vs. vigorously shaken cultures; in these, 24570 lost oxidative ability whereas the M-4243 was unaffected. EDTA has a similar but less dramatic effect on ANS binding: decreasing the ANS binding on 24570 cells at pH 3.3 to a much lesser extent than M-4243. The combination of pH 3.3, EDTA, and vigorous shaking gave the most consistent and largest difference between the strains, but it would seem that EDTA may not be absolutely necessary in order to differentiate between virulent and avirulent strains by this technique.

Using this particular fluorescent probe technique with ANS, we have looked at a variety of Shigella strains with the results obtained in table 3. Most of the Sh. flexneri strains appear to follow the pattern of the M-4243 to 24570 mutation. The X-16 is a hybrid between E. coli, W1895 and the M-4243 which has lost its ability to survive in the gut but has retained its ability to penetrate intestinal epithelial cells. Like its virulent parent, it binds ANS much less than the avirulent, non-penetrating 24570.

The S. Johnson strain is a virulent revertant of strain 24570. It binds ANS far less than 24570, thus resembling the M-4243. The 1 b strains seem to follow this pattern as well, except for strain 228-10, which, although virulent, binds ANS very poorly. However, Kerekes' comments about 1 b strains and agglutinability may apply to ANS binding as well. Sh. flexneri 3 strains seem to have depressed binding characteristic relative to Sh. flexneri 2a strains, but the avirulent strain binds much more than the virulent strain which doesn't appear to bind at all. The 6 serotype pairs is also similar to 2a.

Table 2

Conditions Affecting Binding of ANS

Culture Conditions [1]			M 42 43 [2]	245/0 [2]
pH	EDTA	shaking	fluorescence units / 10^8 cells	
7.0	-	-	30	0
7.0	-	+	13	15
7.0	+	-	44	17
7.0	+	+	19	0
3.3	-	-	602	547
3.3	-	+	177	500
3.3	+	-	268	429
3.3	+	+	125	667

1. Bacteria were grown overnight in Luria broth + 5nM Ca^{++} either in test tubes about 3/4 full growing in static culture or in the same media, vigorously shaking in Erlenmeyer flasks.

2. Fluorescence emission was measured at 470nm with excitation at 360nm; possible experimental error of $\pm$ 20.

Table 3

ANS-binding by Shigella Strains

Serotype	Description	Penetration	Fluorescence
2a	24570	+-	584,635,667
2a	M4243	+	246,188,125
2a - X-16	E. coli hybrid	+	32
2a	S. Johnson - revertant of 24570	+	227
1b	M25-8	+	36,41
1b	2381 0 /124	-	302,374
1b	M-52-994/8f	+	41,25
1b	238-10	-	9,50,52
3	3-50/15	-	162,102
3	J-17-B	+	2
4	M-16-94-0	-	399,147,227
5	M90TX Formal 0	-	80
6	1106 Smith	-	434,206,188
6	CCH0-60	+	37
S. dysenteriae	A-1-38T 18T invades, toxin	+	401,375
S. dysenteriae	725-78 invades, no toxin	+	165,140
S. dysenteriae	A-1-38 18 0 no invasion, toxin	-	417,400
S. dysenteriae	735-19 no invasion, no toxin	-	278,260

Studies of S. dysenteriae strains have just begun and preliminary results are given. Apparently, the production of toxin is associated with good ANS-binding which masks differences in the ability to penetrate host cells. When toxin is not produced, the characteristic greater ANS binding of the avirulent strain is observed, but in this one experiment, the difference is not as dramatic as in the S. flexneri 2a strains. Another experiment was performed with similar relative differences, but lower absolute overall binding by all strains.

These experiments appear to support the use of ANS as a tool to distinguish invasive from non-invasive strains of Shigella, but many more experiments remain to be done to establish the limitations and extensions possible with this approach. Perhaps, even more important is the underlying alteration which links all these different Shigella strains. Not mentioned before is the fact that avirulent, non-penetrating mutants can be due to several different alterations in the cell wall. We have shown that the M4243 to 24570 mutation is associated with a loss of glycerol kinase. The S. flexneri 6 strains are both glp K positive and thus represents a different mutation. Similarly the kcp A mutation found by Formal et al (1971) also affects the ability of S. flexneri 2a to invade intestinal cells.

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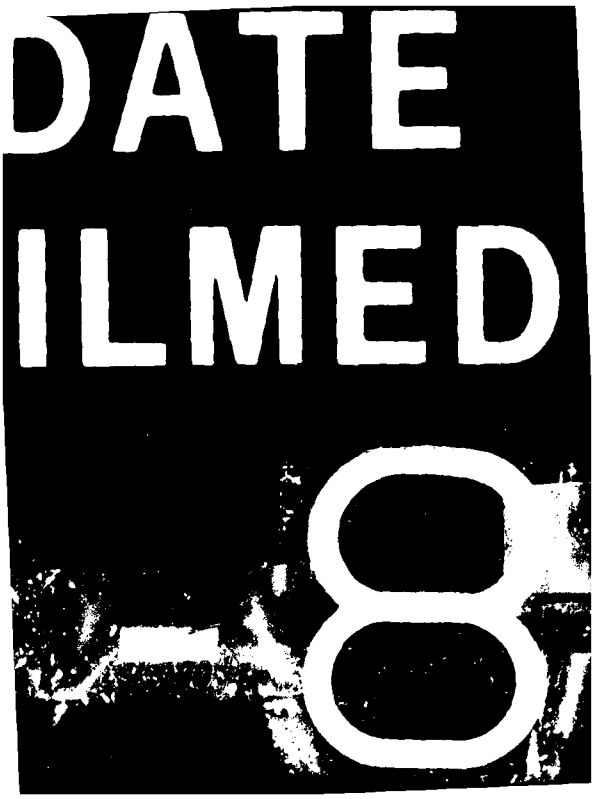
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